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PRACTICAL METHODS IN THE PURIFICATION OF OBLIGATE ANAEROBES

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Nearly every one agrees that many of the descriptive data in the older publications on obligative anaerobes were vitiated by impurity in the cultures examined. While care was usually exercised that no aerobic forms were present, no doubt many cultures have been described as single species in cases where two or more obligate anaerobes were associated. This fact has been responsible for much of the past and some of the present confusion in the literature on the taxonomy of this group.

Whatever method is used to limit a bacterial culture to anaerobes, there is no justification for the asumption of purity of species therein. Until reasonable assurance can be given that a culture does not contain two or more species, differential tests are of no value farther than to show the possible presence or absence of certain organisms with outstanding peculiarities. Thus the production of tetanus in susceptible animals by injection of a toxic filtrate or culture, or of botulism by feeding, may indicate the presence of these organisms but does not prove the purity of the culture. Where special organisms are being looked for, such tests should be made with enriched cultures previous to attempted isolation; but the folly of recording metabolic properties of cultures not consciously and carefully purified is obvious.

It is needless to dwell on this point at length; most of the recent investigators have recognized the necessity of certain purity in anaerobic as well as in aerobic cultures.[1] As Fildes[2] has stated, "the whole problem of anaerobic growth centers around the isolation of pure cultures." The great majority of "new" methods for the culti-

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[1] Meyer, Jour. Infect. Dis., 1915, 17, p. 458. McIntosh and Fildes, Lancet, 1916, 1, p. 768. Henry, Brit. Med. Jour., 1917, 1, p. 763 and p. 806. Donaldson, Lancet, 1917, 1, p. 82. Wilson and Steer, Brit. Med. Jour., 1918, 2, p. 568. Stoddard Jour. Am. Med. Assn., 1918, 70, p. 906. Weinberg and Seguin, La gangrène gazeuse, 1918; Hemple, Jour. of Hyg., 1918, 17, p. 13.

[2] Med. Research Com., Spec. Report Series 12, 1917.

vation of obligative anaerobes have had the goal of surface cultures with a view to facilitating isolation.

There is still a great deal of divergence of opinion as to the best method of securing isolated cultures of unquestioned purity. Thus Weinberg and Seguin,[1] than whom none has been more successful in the study of obligative anaerobes, have used the deep culture method throughout as a means of isolation; the English investigators, McIntosh and Fildes,[1] Hénry,[1] Wilson and Steer,[1] Adamson,[3] and the members of the Medical Research Committee led by Bullock,[4] have used surface culture methods. In this country, Meyer,[1] Dickson and Burke [5] used the deep culture method successfully, while Stoddard [6] has followed the English in the use of surface methods. In addition to the above, there have been scattering attempts to utilize purely microscopic means of isolation.

This paper summarizes certain observations in the isolation of a considerable number of cultures among which have been identified B. sporogenes, 18 strains; B. welchii, 3 strains; B. tetani, 1 strain; B. botulinus, 3 strains; B. chauvaei, 1 strain; vibrion septique, 7 strains; and several others not yet completely identified.

INITIAL CULTURE AND ENRICHMENT

The usual occurrence of mixed flora of aerobes and anaerobes in nature makes some method of enrichment of the latter generally desirable as a preliminary step to complete elimination of the aerobes and isolation of the anaerobes, especially in those cases in which growth may have come to a standstill, as in canned goods, or in soil or water. Where active pullulation is in progress, one may proceed at once to dilution and isolation. Thus McIntosh [7] advocated the direct streaking of pus from war wounds, when possible, as giving a more nearly correct estimate of the proportion of various organisms originally present. But the usual occurrence of facultative anaerobes is likely to make actual isolation a complicated problem.

The alternate plan of enrichment previous to isolation permits a choice of methods from a considerable variety of procedures. The constricted tube with marble seal[8] using glucose broth (P_H 7-8) as a medium is preferable for this purpose from the standpoints of simplicity and efficiency.

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[3] Jour. Path. and Bacteriol., 1919, 22, p. 345.

[4] Med. Research Com., Special Report Series 39, 1919.

[5] Jour. Am. Med. Assn., 1918, 71, p. 518.

[6] Lancet, 1919, 196, p. 12; Jour. Exp. Med., 1919, 29, p. 187.

[7] Med. Research Com., Special Report Series 12, 1917.

Inoculation should be immediately preceded by heating the medium for a few minutes in a bath of boiling water and cooling to 50 C. The material under examination should be introduced partly below the seal and partly above.

The temperature of incubation should be 37 C. usually; even B. botulinus, which, according to most of the older authors, grows better at 20-25 C., in our experience grows more rapidly and heavier at 37 C. and produces strong toxin at that temperature also.

Growth of anaerobes in the constricted tube naturally starts below the seal. But as soon as the evolution of gas begins even cultures of anaerobes free from aerobes are likely to produce turbidity above the seal as well as below due to the fact that each bubble of gas, whether it be carbon dioxide, hydrogen, methane, hydrogen sulphide, or a mixture of these and others, acts as a vacuum so far as other gases, including oxygen, are concerned. Each escaping bubble carries its load of oxygen, until the medium becomes anaerobic throughout. This explanation accounts satisfactorily also for the ease with which merely deep tubes of medium may be made to yield anaerobic growth, especially if previously boiled out, or aerophilic organisms be present, or a nidus for the initiation of growth in the depths be present. Late growth near or even on the surface does not therefore necessarily imply the presence of aerobic organisms.

Twenty-four hours' incubation are generally sufficient, but in some cases 48 are necessary, and complete sterility cannot be assured within a week at least. In making a sterility test of biologic products such as serums and vaccines, which, incidentally, is the specific purpose for which the constricted tube was invented, an incubation period of one week is recommended by the Hygienic Laboratory of the United States Public Health Service. In certain instances in which the bacterial cells have been subjected to injury, as, for example, in the heating of bacterial vaccines, concentration of tuberculin, or canning processes (Burke,[9] 1919), the period of bacterial lag may be prolonged considerably.

Closely second to the constricted tube for enrichment of anaerobes are deep brain and meat mediums. Introduced by Von Hibler,[10] these mediums were first used extensively in this country by Meyer[1] and during the war they were employed widely in work with anaerobes. Bradley[11] has shown the utility of brain medium in preserving the viability of the meningococcus and Holman[12] has emphasized the value of a cooked meat medium in special and routine work. While the general results with these mediums are similar, as shown in the records of the English writers,[3, 4] who have used meat largely, while the French have used mostly brain, the consistency and whiteness of the brain medium predispose one to its preference. As usually prepared, it consists merely of a mixture of ground sheep or beef brains with water, equal parts, tubed to a depth of 10-12 cm. in culture tubes. Sheep brains are preferable as being less bloody, since it is not customary to stun sheep by a blow on the head before killing; otherwise there is little or no difference as to source. There is a distinct advantage in the addition of a modicum of glucose to stimulate rapid growth and gas formation, but an excess is to be avoided as prohibiting sporulation (generally) and interfering with the blackening of the medium, which is characteristic of the putrefactive anaerobes and therefore of diagnostic value. An addition of peptone is also advantageous in stimulating early development and intensifying the pigmentation mentioned above. The following is the present method of preparation:

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[8] Univ. Calif. Pub. in Path., 1915, 2, p. 147.

[9] Jour. Bacteriol., 1919, 4, p. 555.

[10] Ueber die pathogenen Anaeroben, 1908.

1. Boil sheep brains with an equal volume of distilled water.
2. Decant water (save) and press brains through a potato ricer.
3. Add decanted water, 2% peptone, and 0.1% dextrose.
4. Tube by punching through the filling funnel with a glass rod, filling tubes about half full.
5. Sterilize intermittently in the Arnold sterilizer. Five daily runs of 30 minutes each are recommended.

Sterilization in the autoclave has usually been a failure owing to the tendency of the medium to splatter up onto the plugs. It should be and remain nearly white with a clear supernatant liquid above the brain substance. Growth is indicated by turbidity and in many cases by production of gas. The presence of glucose provides for those organisms, such as the tetanus bacillus, which appear to sporulate better in its presence, but the small amount used is exhausted shortly so that those organisms which would be prevented from sporulation by an excess retain their vitality for considerable periods of time. Brain medium therefore serves for storage as well as for enrichment.

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[11] Jour. Am. Med. Assn., 1918, 70, p. 1816.

[12] Jour. Bacteriol., 1919, 4, p. 149.

Milk favoring the development of B. welchii has been a favorite medium for enrichment of material from war wounds. Defensible in that case, since the principal other pathogenic anaerobes in war wounds, excepting B. tetani, but including the vibrion septique and B. oedematiens, are lactose fermenters, the general use of milk might exclude, or at least suppress, certain nonlactose fermenting microorganisms.

English writers have developed the idea of selective enrichment quite fully and have shown that the predominating flora of a mixed meat culture, for example, varies from day to day in a regular order, when the material has come from a badly infected wound. There are 3 periods, each marked by certain metabolic and morphologic characteristics of the predominating organisms. At the end of the first 24 hours, organisms of the fermentative type, such as B. welchi, B. fallax, and vibrion septique predominate; in another day putrefactive organisms, such as B. sporogenes, are in the ascendency, while after some days of further incubation, nonfermentative bacteria without marked putrefactive properties such as B. tetani appear. It happens that the spores of the organisms of the first two periods are central or subterminal while those of the last are terminal, so that the metabolic characteristics of the changing flora are correlated with distinctive morphologic properties.

These facts have found their application in the provision of carbohydrate mediums for the enrichment of fermentative bacteria, and of sugar-free protein rich mediums for putrefactive organisms.

Some of the English scientists have advocated also the use of animals for enrichment. When a single pathogenic anaerobe is known to be associated with nonpathogenic species the latter can often be eliminated by animal passage through normal animals susceptible to the first. For a mixture of pathogenic organisms it is stated that actively or passively immunized guinea-pigs or mice can be used as a means of selective elimination.

The writer has had little occasion to test the use of animals in this manner; some attempts to eliminate the hay bacillus from a culture of B. welchii were less successful than the use of inhibitive dyes shortly to be described. At any rate the use of selective mediums and selective animals should not be thought of as means of isolation; the English writers have made it clear that these are only preparatory to plating; they are, in other words, merely enrichment methods.

ELIMINATION OF AEROBES

Here again the question might be raised as to the propriety of postponing the isolation process. Ideally desirable, it is practically difficult to secure pure cultures of anaerobes from cultures mixed with aerobes previous to the employment of certain procedures for elimination, or at least suppressing the latter. After one has secured the primary culture one may begin at this point the separation of the anaerobic from the aerobic examination. If the latter is important, suitable solid medium, e. g., blood-agar plates or slants in the case of pathologic materials, should be streaked out for the isolation of the aerobes; otherwise, a plain agar slant is usually sufficient to indicate the possible presence of aerobes and their general nature, as shown by incubation at 37 C. and subsequent Gram stain. In case no growth occurs aerobically on solid mediums the culture can be assumed to be free from aerobes. Such a test for the exclusion of aerobic contamination should be made frequently in anaerobic work and in every case in which important conclusions are to be drawn as a criterion of aerobic contamination.

Two general categories of methods are available for the elimination of aerobes from cultures containing anaerobes, namely, selective heating and selective cultivation in special mediums.

Selective heating is applicable to sporulating anaerobes only in the presence of nonsporulating contaminants. This limitation necessitates the use for most anaerobes of culture medium having less than an excess of fermentable carbohydrate, as, for example, brain medium, or the alkaline egg medium of Robertson,[13] in order to insure sporulation if possible. A temperature of 85 C. for 10-15 minutes is usually sufficient to destroy all vegetative forms; occasionally a higher temperature or longer exposure may be required. To avoid loss of the initial culture subcultures should be taken before as well as after heating.

When aerobic spores are present, selective heating is usually a failure, since most of the anaerobic spores are less resistant, with the possible exception of B. sporogenes. For the elimination or suppression of these, selective culture methods may be utilized.

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[13] Jour. Path. and Bacteriol., 1916, 20, p. 237.

One of these consists in the rapid growth and frequently repeated transfer of cultures of saccharolytic organisms, such as B. welchii in glucose broth as advocated by Taylor[14] and Tullock.[15] Tullock has applied the same idea in the selective cultivation of nonfermentative organisms such as B. tetani in medium fermented out with B. sporogenes.

The use of certain tri-amino-tri-phenyl-methane dyes, such as gentian violet and crystal violet, for the elimination of hay bacilli from cultures of obligative anaerobes,[16] is also recommended. The following is a transcript of a characteristic record showing how this method is operated:

B. sporogenes No. 66 contaminated with aerobic spores, 2/26/20 Glucose broth G.V.

- Gas
  - Glucose broth G.V. gas
    - Glucose broth G.V.
      - Glucose broth G.V. gas
        - Glucose broth G.V. gas
        - Deep meat....... —gas
        - Plain agar. — — —
      - Deep meat.......++gas
      - Plain agar......... ++
    - Deep brain..........gas
    - Plain agar aerobic.. +
  - Deep brain........+gas
  - Plain agar aerobic +

Culture now considered aerobe free.

The method can nearly always be made to work sufficiently to eliminate completely all the aerobic spores, but if it cannot, there is a great advantage in using it as a preliminary suppressive measure immediately preceding isolation.

In all selective methods there is the possibility of losing desirable organisms from the culture, and one should take the precaution of holding stock cultures of the initial culture until it is certain that none of the organisms it contains has been missed.

PURIFICATION

Purification may be accomplished frequently from original material, even that containing aerobes, but if the attempt is not successful immediately some of the anaerobe enrichment and aerobe suppression procedures just described should be carried out.

SURFACE METHODS OF ISOLATION OF ANAEROBES

The writer was engaged intermittently during five years previous to 1917 in testing various of the described methods, with indifferent success, until he was led to the following modification of Wright's[17] method: Rabbit and horse blood agar slants prepared from the usual meat infusion nutrient broth with 3% agar plus 5-10% sterile defibrinated blood have been used mostly. The tubes should be plugged with absorbent cotton. The slanted surface should be moderately dry at the time of inoculation although a small amount of water of condensation in the lower angle of the slant does no harm if precautions be taken to prevent its flowing over the inoculated surface on inversion of the tubes. Inoculation may be from any suitable liquid culture; we have used mostly glucose broth, constricted tube cultures or the supernatant fluid portion of deep brain cultures, avoiding, in the latter case, the transfer of solid particles. The slants are streaked by beginning at the bottom, diluting in the water of condensation (or hysteresis) if any, and spreading over the surface of the slant by slowly withdrawing the loop, at the same time making rapid transverse movements over the agar. Replacing the cotton plug in the usual manner it is then cut off squarely with the scissors and pushed into the tube so that its top is about 1.5 cm. below the mouth of the tube, and it does not touch any part of the slanted medium. The space above the cotton plug is then filled with pyrogallic acid crystals (0.5-1.5 gm.) and a rubber stopper selected of such size as to plug the opening snugly. With everything ready, the tube is then held in the left hand with the inoculated surface toward the right; 1-2 c c of 10% lye are poured on the crystals from a bottle and the rubber stopper placed in position. The tube must be inverted at once and without allowing the moisture in the tube to flow over the inoculated surface, that is, it must be made to flow down into the stopper over the glass surface of the tube in order to prevent spreading colonies. The obvious purpose of inverting the tubes is to avoid soiling the culture with the lye-pyrogallol solution.

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[14] Jour. Path. and Bacteriol., 1916, 20, p. 384.

[15] Med. Res. Com., Special Report Series 39, 1919.

[16] Jour. Am. Med. Assn., 1919, 72, p. 61.

Incubation at 37 C. usually gives growth within 24-48 hours; yet certain cultures of B. putrificus from Professor Sturgis of Yale University rarely showed evidence of growth in less than 5 days, at which time one could rely fairly regularly on finding an abundant culture. This interesting fact might be interpreted as indicating a somewhat lower oxygen requirement on the part of this organism, which was met in this method only by a somewhat longer prolonged operation of the oxygen absorbing mixture.

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[17] Jour. Bost. Soc. Med. Sci., 1900, 5, p. 114; also Centralbl. f. Bakteriol., 1, 1901, 29, p. 61.

Satisfactory surface growth can be secured in approximately nine trials out of ten. Well separated colonies are a matter of experience in dilution as they would be under aerobic conditions and have been obtained in about three fourths of the trials. Attempts to secure possible latent nongrowing bacilli from the bare spaces between well separated colonies were never successful. Isolated colonies can be picked into deep brain medium or glucose broth in constricted tubes with impartial success; only occasionally does a picked culture fail to grow.

With this method some 40 cultures now under study in this laboratory were purified. The details will be discussed in connection with the description of the cultures. Reliance was never placed in a single streaking and picking for isolation, but the purification has been repeated in each instance at least 3 times and in some cases 8 times before purity was finally assumed. Meticulous care to detect the persistence of impurity or the appearance of a new contamination was exercised constantly. This procedure seems as reliable as the repeated streaking and picking of aerobes under analogous conditions and justifies the opinion that the isolation of these cultures was thoroughly complete.

But the finding of two cultures, one of B. welchii and one of B. tetani, apparently contaminated anaerobically after they were supposedly purified as above, served to point out a possible fallacy in all methods of surface culture isolation of obligative anaerobes. This conviction was supported by the observation of a culture known to be impure in which one organism grew for a time in a very narrow range of oxygen tension only, as indicated in deep glucose agar. The method of isolation from deep glucose shake cultures will be discussed shortly in order to indicate some possible theoretical limitations in all cultural methods of isolation, but which apply especially to surface culture methods. However, evidence is lacking to show that the considerations to be named are of anything more than theoretical importance; at any rate, they must appear only rarely. The contamination found in the tetanus and Welch bacillus cultures mentioned above was not attributable to any fault of isolation since earlier duplicate cultures in both cases made subsequent to isolation failed to show the impurity, which was probably due to contaminated medium. Furthermore, a rigid examination of the remaining cultures since isolation has failed to show a single instance of anaerobic contamination attributable to faulty isolation.

DEEP CULTURE METHODS OF ISOLATION

One of the oldest methods of isolating anaerobes and at the same time one of the best, if not actually the very best, the deep culture method of Hesse,[18] has been more widely used, perhaps, than any other cultural means of anaerobe cultivation and isolation. It utilizes ordinary culture tubes, or better still, the rubber stoppered tubes of Burri,[19] which may be filled to a depth of 10-12 cm. with either agar or gelatin medium. The writer has used mostly 1% agar in meat infusion broth adjusted automatically to a constant alkalinity by means of magnesium carbonate in excess which is allowed to settle out of suspension before the agar cools, by holding in the Arnold sterilizer for an hour or two at 100 C. After cooling, the container, usually a 1,000 c c conical graduate, is inverted on a clean surface to eject the solid mass and that portion containing the sedimented excess of magnesium carbonate and precipitate is cut off, leaving the remainder clear and with a nearly constant reaction of P_H equal approximately to 8.1, which is quite suitable for the growth of most sporulating anaerobes. Excellent gelatin can be made in the same manner. It has been customary to add 0.2% to 1.0% glucose to the agar, to stimulate growth, at this point. Glucose should not be heated along with the other ingredients in the presence of magnesium carbonate, owing to excessive hydrolysis which is likely to occur. If excessive splitting of the medium occurs during growth of the organisms, the reduction of the glucose content to a minimum goes far to avoid the difficulty and is preferable to the use of potassium nitrate as advocated by Veillon and Maze.[20]

With young brain or glucose broth seed cultures one can usually secure suitable dilutions to provide well separated colonies by rinsing the inoculated loop in a tube of sterile water. It has seemed unnecessary to shake mixed cultures with sand as advocated by Stoddard,[1] though it is conceivable that circumstances might arise in which this would be desirable. Ordinarily the sporulating anaerobes develop in glucose broth or in the supernatant fluid of brain mediums in a homogeneous suspension; the individual bacteria fall apart readily and do not tend to form conglomerate masses which would be likely to contain more than one species. Inoculation should be made in boiled mediums cooled down to 40-50 C., care being taken to distribute the organisms well by rotation of the inoculated tube previous to solidification.

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[18] Deutsch. med. Wchnschr., 1885, 11, p. 214.

[19] Centralbl. f. Bakteriol., II, O., 1902, 8, p. 533.

[20] Comptes. rend. Soc. biol., 1910, 68, p. 112.

Most cultures yield growth within 24-48 hours' incubation at 37 C. If well separated colonies appear, the tube can be cracked near the base with a file and encircled with a hot rod or electric wire to complete the fracture, or, in the case of Burri's tube, the rubber stopper is removed, and the agar plug is pushed out from the other end into a sterile dish. It is now sliced transversely into disks 4-6 mm. thick, with a scalpel boiled in 1-2% lye, thus exposing to view the colonies embedded in the medium. The intervening agar is partly seared away from such of these as it is desired to cultivate, as advocated by Burke,[21] and the selected colony removed en masse with a slender picking needle to deep brain, glucose broth, or other suitable medium. The searing of the agar is to be considered an important part of this method as avoiding the transfer of organisms other than those inclosed in the colony itself. This procedure, especially if several times repeated, gives results equal to those obtained by surface culture methods.

During the use of the deep culture method an observation was made which would have been overlooked in Wright's method or any other surface culture method, and as such would constitute a serious drawback to the use of surface culture methods for isolation, if it occurred frequently, which is doubtful.

A certain culture, later shown to contain B. sporogenes, on diluting in glucose agar, presented, after incubation, two distinct types of colony, one an obligative anaerobe "Y" growing only in narrow range (about 5 mm.) at a constant depth below the surface, i. e., 3 cm.; the other a facultative anaerobe "X," developing throughout the depth of the medium. No colonies of "X" were ever observed to invade the range of "Y." The situation is diagramed roughly in the illustration. Several points arise: First, "X" colonies picked into deep brain always developed as a mixture with "Y"-like organisms, owing doubtless to latent "Y" organisms lying in the picked colony. It was easy to determine the admixture because "X" proved to consist of Staphylococcus albus, while "Y" consisted of rods. In case "X" colonies had ever been found lying within the range of "Y," each might have been secured free from the other; there was no difficulty in picking "Y" free from "X" the first time. As it turned out, "X" proved to be aerobic and only facultatively anaerobic so that "Y" could be eliminated easily by repeated surface culture; in case "X" had been an obligative anaerobe there would have been more difficulty in securing a pure culture. A similar instance of an organism with a narrow range of oxygen tension tolerance occurred in the case of an obligatively anaerobic micrococcus recovered from the nasal washings of a patient with acute rhinitis.[22]

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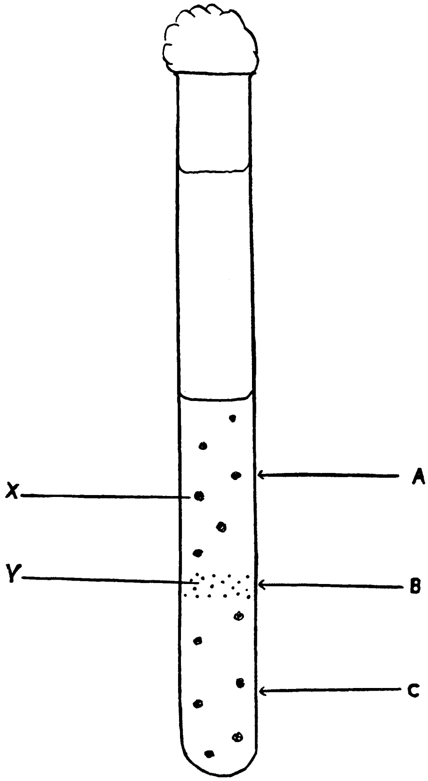
[21] Jour. Bacteriol., 1919, 4, p. 555.



A tube of deep glucose agar containing two types of colony; one grew in a narrow range of reduced oxygen tension, the other grew throughout the tube.

If one examines the possibilities of such a culture in Wright's method, he may note that the oxygen tension is reduced probably in a given instance to a definite, but unfortunately, unknown point at which a balance occurs with the absorbing mixture of alkaline-pyrogallol. This point probably varies from time to time according to the strength of reagents used, size of air space, etc. Suppose the oxygen tension reduction corresponds to a point "A" or "C" in the deep agar; no colonies of "Y" would appear. If it should happen to strike a point analogous to "B" and conditions are similar to those that seemed to exist in the deep tube, no colonies of "X" would appear, though the reason for their failure to appear in the range of "Y" in either case is somewhat unclear, unless it be a question of antagonism, for which there is no direct evidence.

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[22] Hall, Unpublished manuscript.

These somewhat theoretical considerations led to a reconsideration of microscopic methods of isolating single organisms as means of avoiding the above mentioned difficulties.

MICROSCOPIC METHODS OF ISOLATION

The preparation of pure cultures in general has been reviewed carefully by Jörgensen,[23] who has pointed out the value of direct optical observation of the development of a single cell completely isolated, a method quite feasible with yeasts and molds but much less so with bacteria on account of their small size.

As early as 1821, Ehrenberg followed the germination of certain fungi microscopically, as did also Mitscherlich in 1843, Kützing in 1851, and Schülze in 1860, that of yeast cells, and Tulasne in 1861, and De Bary in 1866, that of bacterial spores. In 1881, Brefeld invented the moist chamber for cultivation of organisms under microscopic observation which was especially emphasized by Hansen as a means of purifying yeasts. Braatz[24] devised, in 1890, a special slide for the cultivation of anaerobes over alakline-pyrogallol in hanging drops, and the hanging drop method of isolation was further developed by Lindner in 1893. The method consists in a preliminary dilution, in drops of sterile medium hanging from the under side of a sterile cover, such that each drop contains approximately one cell only; those drops which meet this expectation, as shown by microscopic examination, are marked and transferred, after incubation, as pure cultures. A modification aiming to facilitate the detection of organisms in such drops was the use of India ink, as suggested by Burri.

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[23] Micro-Organisms and Fermentation, 1911.

[24] Centralbl. f. Bakteriol., 1890, 8, p. 520.

A modern development of microscopic methods of isolation is found in the technic of Barber,[25] which involves the use of a capillary pipet in picking single organisms out of a diluted suspension under the microscope. Donaldson[1] appreciated the theoretical advantages of optical isolation of anaerobes, but his recognition of the technical obstacles led him to the advocacy of a method of dilution involving a hemocytometer count followed by culture of amounts theoretically such as to distribute the organisms singly in separate tubes—virtually a return to the old dilution methods of Lister and Hansen in use prior to the invention of solid mediums by Koch.

The cultivation of aerobic bacteria from single cells has been carefully investigated recently by Hort,[26] who refers to six distinct methods. Hort considers the optical difficulties and the use of liquid medium in these methods insurmountable obstacles to the continuous observation from single cell to colony which he considers necessary. His own method of covering thinly spread dilutions on agar slabs with cover slips for observation might be successful for anaerobes, but this has not been claimed.

The writer's efforts have been restricted to Barber's method. After seeing Professor Barber isolate a pair of pneumococci, one cannot doubt the general efficacy of the procedure in the hands of so skilful a master of technic. My own persistent attempts have never met with anything but the most dismal failure in the use of his method, but Hempl[1] has claimed to use Barber's method quite successfully.

In conclusion, the method of culture in deep glucose agar seems quite satisfactory, especially if repeated isolation is practiced with each culture. Actually, one usually secures a pure culture the first time.

SUMMARY

This article calls attention to the relatively recent appreciation of the failure of some early investigators to secure unquestionably pure cultures of obligative anaerobes.

The methods of initial culture and selective enrichment are discussed and certain observations are noted on the elimination of aerobic contaminants through selective heating and selective cultivation.

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[25] Jour. Infect. Dis., 1911, 8, p. 349; Phil. Jour. Sc., 1914, B9, p. 307.

[26] Jour. Hygiene, 1920, 18, p. 307.

Certain limitations on cultural methods of isolation, especially surface methods, are suggested, and a critical review of the present status of surface culture methods, deep culture methods and microscopic methods of isolation, is given.

It is concluded that the method of isolation by deep cultivation in glucose agar is most practical.